Fungal diversity and specificity in Cephalanthera damasonium and C. longifolia (Orchidaceae) mycorrhizas

Pecoraro, L., Huang, L., Caruso, T., Perotto, S., Girlanda, M., Cai, L., & Liu, Z-J. (2017). Fungal diversity and specificity in Cephalanthera damasonium and C. longifolia (Orchidaceae) mycorrhizas. *Journal of Systematics and Evolution*, *55*(2), 158-169. https://doi.org/10.1111/jse.12238

Published in:
Journal of Systematics and Evolution

Document Version:
Peer reviewed version

Queen's University Belfast - Research Portal:
Link to publication record in Queen's University Belfast Research Portal

Fungal diversity and specificity in *Cephalanthera damasonium* and *C. longifolia* (Orchidaceae) mycorrhizas

Lorenzo Pecoraro[1,2,3,*], Laiqiang Huang[2], Tancredi Caruso[4], Silvia Perotto[5], Mariangela Girlanda[5], Lei Cai[3], Zhong-Jian Liu[1]

[1] *Shenzhen Key Laboratory for Orchid Conservation and Utilization, The National Orchid Conservation Center of China and The Orchid Conservation and Research Center of Shenzhen, Shenzhen, China*

[2] *Center for Biotechnology & BioMedicine and Division of Life & Health Sciences, Graduate School at Shenzhen, Tsinghua University, Shenzhen, China*

[3] *State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China*

[4] *School of Biological Sciences, Queen's University of Belfast, Belfast, Northern Ireland*

[5] *Department of Life Sciences and Systems Biology, University of Turin, Turin, Italy*

*Corresponding author: Lorenzo Pecoraro (address: The Orchid Conservation and Research Center of Shenzhen, Wangtong Road 889, 518114 Shenzhen, China; e-mail address: lorenzo.pecoraro@gmail.com)

Running title: **Mycorrhizal associations in *Cephalanthera* species**

Abstract

Orchids depend on mycorrhizal fungi for their nutrition, at least in the early stages of their growth and development and in many cases throughout the life. In spite of the increasing number of studies describing fungal diversity in orchids, there is still more to be learnt about the identity of fungal partners and specificity in orchid mycorrhizal associations. We investigated the fungal communities associated with the roots of *Cephalanthera damasonium* and *C. longifolia* adult plants, using morphological methods and fungal ITS-DNA PCR amplification, cloning and sequencing. A range of fungi belonging to Basidiomycota and Ascomycota was uncovered in the roots of the two investigated orchid species, showing a low degree of mycorrhizal specificity. At least 11 fungal taxa, including *Cenococcum geophilum*, *Ceratobasidium* sp., *Exophiala salmonis*, Hymenogastraceae, and Sebacinaceae colonized *C. damasonium* roots, while about 9 fungal types, such as *Bjerkandera adusta*, *Phlebia acerina*, Sebacinaceae, *Tetracladium* sp., and *Tomentella* sp. associated with *C. longifolia*. Phylogenetic and statistical analyses indicated significant differences in the fungal communities associated with the two studied *Cephalanthera* species, as well as distinct mycorrhizal partners associated with each orchid plant. Our results strongly suggest that both *C. damasonium* and *C. longifolia* are generalist in their mycorrhizal associations.

Keywords Ascomycetes; basidiomycetes; fungal symbionts; mycorrhizal specificity; Orchidaceae; orchid mycorrhiza.

1 Introduction

Orchid mycorrhiza is recognized as a distinct type of endomycorrhiza where individual fungal hyphae cross the orchid cell wall by simple penetration and form pelotons (intracellular coils) in the cortical tissues of protocorms, roots, tubers and rhizomes, in close connection with the invaginated plant plasmalemma (Rasmussen, 1995; Smith & Read, 2008).

As well as from the anatomical point of view, orchid-fungus associations are unique in their physiological and nutritional aspects. Indeed, the great majority of plants associate with soil fungi that usually exchange mineral nutrients for plant carbon (Smith & Read, 1997), whereas orchids are dependent upon mycorrhizal fungi for the provision of carbohydrates, at least in the early development. Thus, the polarity of carbon movement in orchid mycorrhiza, occurring from the fungal partner to the plant, is inverted as compared with the general condition existing in the other mycorrhizal types (Smith, 1967). Moreover, the relationship between orchids and symbiotic fungi is considered a one-way system in favour of the plant (Merckx et al., 2009; Rasmussen & Rasmussen, 2009), instead of a mutualistic association as in the arbuscular mycorrhizas and ectomycorrhizas, with a very few exceptions (Cameron et al., 2006).

This fungus-dependent life style, called mycoheterotrophy (Leake, 1994; Bidartondo & Read, 2008), is a necessity for all orchid species, during the achlorophyllous protocorm stage. At the adult stage, most orchids develop a photoassimilating apparatus and become autotrophic, although they continue to rely on their fungal associates for water and mineral salts (Liebel et al., 2010; Girlanda et al., 2011; Jacquemyn et al., 2011a). On the contrary, some orchid species, growing in forest habitats, remain dependent on fungal sugars even at maturity. Based on their carbon nutrition, these forest species can be divided into two physiological types (Dearnaley & Bougoure, 2010). Fully mycoheterotrophic orchids are achlorophyllous and completely depend on mycorrhizal fungi for their carbon, throughout their life cycle (Bidartondo et al., 2004; Roy et al., 2009). Partially mycoheterotrophic orchids, are green and perform photosynthetic carbon fixation, but also obtain additional carbon from their mycobionts with varying extents, depending on light availability (Preiss et al., 2010). All studied achlorophyllous orchids associate specifically with narrow phylogenetic fungal groups (Taylor et al., 2002; Selosse & Roy, 2009), while partially mycoheterotrophic orchids, using this newly discovered, dual nutritional strategy, also called mixotrophy (Selosse et al., 2004), show a variable level of mycorrhizal specificity (Julou et al., 2005; Girlanda et al., 2006).

The genus *Cephalanthera* Rich. mainly belongs to the latter trophic group. This orchid taxon comprises a total of 15 species with a mostly Eurasian distribution (Delforge, 2006). Only one species *C. austiniae* (A. Gray) A. Heller, characterised by permanent loss of chlorophyll and strong leaf reduction, has been found in North America (Colemann, 1995). Seven species are the European representatives of the genus (Delforge, 2006). Among them, *C. damasonium* (Mill.) Druce and *C. longifolia* (L.) Fritsch have been found to obtain carbon both through photoassimilation and from their fungal root associates (Gebauer & Mayer, 2003; Julou et al., 2005; Abadie et al., 2006), thus indicating that these species constitute typical examples of mixotrophic orchids. These two phylogenetically close orchid species (Bateman et al., 2004) are rhizomatous plants with perennial roots, summer-green and overwinter underground, growing in broadleaved, coniferous, and mixed forests, often colonizing shadowy forest edge, between shrubs and trees (Rossi, 2002; Delforge, 2006). Previous studies have focused on the identification of their mycorrhizal fungi. Fungal diversity in *C. damasonium* has been assessed in three different works carried out in France, Germany, and Hungary showing that this orchid associates with a variety of both basidiomycetes and ascomycetes (Bidartondo et al., 2004; Julou et al., 2005; Illyes et al., 2010), while Abadie et al. (2006) and Liebel et al. (2010) have respectively demonstrated that *C. longifolia* associates with several fungal taxa, such as Thelephoraceae and Sebacinaceae, in Estonia, whereas *Hebeloma*, *Russula*, and *Tomentella* are the main mycorrhizal fungi in the same orchid species collected in Italy. All these studies have reported on mycorrhizal diversity in only one of the two *Cephalanthera* species, analysing a small number of samples (four *C. damasonium* individuals in Julou et al. (2005) and Bidartondo et al. (2004), only two *C. longifolia* adult plants in Liebel et al. (2010)), from a single sampling site. Very different environmental conditions have been described for the sampling sites investigated in the above mentioned works. These conditions, together with the geographical distance between studied areas, may influence the fungal community in each site (Gale et al., 2010; McCormick & Jacquemyn, 2014), and hence make difficult to understand whether the mycorrhizal diversity up to now reported in *C. damasonium* and *C. longifolia* reflects the ecological preference of the two orchid species or is mainly a consequence of local factors. Therefore, the degree of mycorrhizal specificity in *C. damasonium* and *C. longifolia* is still uncertain. The extent of specificity towards fungi shown by these two orchid species has been previously investigated just in a single work, carried out by Bidartondo & Read (2008). Specificity of orchid-fungus relationships has an important role in orchid biology and conservation (Jacquemyn et al., 2010; Phillips et al., 2011).

Orchid species that establish generalist associations with multiple fungal symbionts may be more adaptive under changing environmental condition or in fragmented habitats than orchids that associate with only a few fungal partners (Swarts & Dixon, 2009). On the other hand, specialist orchid taxa may increase their fitness in specific habitats or under a narrow range of environmental conditions by selecting a specific, highly ecologically competent fungal symbiont (Bonnardeaux et al., 2007), but this high degree of mycorrhizal specificity may be linked to orchid rarity when the fungal partner has a limited distribution (Swarts et al., 2010).

In this study, we analysed fungal diversity and specificity in *C. damasonium* and *C. longifolia* mycorrhizal associations, using morphological and molecular methods. Orchid roots were collected from different sites, both total root DNA and DNA from isolated fungi were extracted and fungal ITS regions were PCR amplified, cloned and sequenced.

First, we investigated for each species whether an association with different fungal partners might occur in different sites. Second, we assessed specificity between the two orchids and their mycobionts by analysing the phylogenetic distance of their fungal associates. Finally, we tested mycorrhizal specificity for the studied orchid species by determining whether they associate with the same fungi when they grow in sympatric populations or they maintain distinct mycorrhizal diversity.

2 Materials and methods

2.1 Study sites and sampling

Cephalanthera damasonium and *C. longifolia* plants were sampled from nine forest edge sites located in three geographically distinct protected areas in Tuscany (Central Italy), “Monte Cetona”, “Monte Penna” (specifically on “Monte Rotondo”), and “Cornate di Gerfalco” Natural Reserves, characterized by the presence of dry calcareous meadow habitats with *Bromus erectus* Hudson dominating among herbaceous plants, surrounded by forests with various broad-lived species such as *Fagus sylvatica* L., *Quercus cerris* L., *Ostrya carpinifolia* Scop., and *Acer* sp.pl. mixed with conifers such as *Pinus nigra* Arnold. The study sites lie on the top of mountains, at an altitude ranging from 755 to 1026 m a.s.l.

The two analysed orchid species were growing together only in one (“Monte Rotondo”) out of the three investigated areas.

During the early summer in 2007 and 2008, at orchid flowering, root samples were collected from a total of eleven individuals for each studied *Cephalanthera* species (six *C. longifolia* individuals in "Cornate di Gerfalco", six *C. damasonium* in "Monte Cetona", and five plants for each species in "Monte Rotondo"). All roots were washed under running water, carefully brushed, and treated in an ultrasonic bath (three cycles of 30 s each) in order to remove soil debris and to minimize the detection of microorganisms adhering to the root surface. Fresh root samples were cut into 1 cm long pieces and their mycorrhizal morphology was observed under a light microscope, on thin cross sections. Root portions exhibiting high fungal colonization were partly processed for fungal isolation and partly frozen in liquid nitrogen and stored at -80°C for molecular analysis.

2.2 Fungal isolation

Fresh orchid root fragments were immediately processed after sampling. Five or six roots per plant were surface-sterilized with consecutive washes of 5% sodium hypochlorite (30 s) and three rinses of sterile water. Ten 3-5 mm long pieces from each root were cultured in malt extract agar (MEA) and potato dextrose agar (PDA) amended with gentamycin (40 mg/l) and/or chloramphenicol (50 μg/ml). Petri dishes were incubated at room temperature in the dark for up to two months to allow the development of slow-growing mycelia.

2.3 Molecular identification of orchid root fungi

Both total DNA from orchid root samples and DNA from isolated fungi were extracted using the cetyltrimethyl ammonium bromide (CTAB) procedure modified from Doyle & Doyle (1990).

Fungal ITS regions were PCR amplified using the primer pair ITS1F/ITS4 (Gardes & Bruns, 1993) in 50 μL reaction volume, containing 38 μL steril distilled water, 5 μL 10 × buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 11 mM $MgCl_2$, 0.1% gelatine), 1 μL 10 mM dNTP, 1 μL of each primer (ITS1F and ITS4), 1.5 U of RED *Taq*TM DNA polymerase (Sigma) and 2.5 μL of extracted genomic DNA at the appropriate dilution. Amplifications were performed in a PerkinElmer/Cetus DNA thermal cycler, under the following thermal conditions: 1 cycle of 95°C for 5 min, 30 cycles of 94°C for 40 s, 55°C for 45 s, 72°C for 40 s, 1 cycle of 72°C for 7 min. The resulting PCR products were electrophoresed in 1% agarose gel with ethidium bromide and purified with the QIAEX II Gel Extraction Kit (QIAGEN) following the manufacturer's instructions.

The purified ITS fragments were cloned into pGEM-T (Promega) vectors that were used to transform XL-2 Blue ultracompetent cells (Stratagene). After transformation, twenty white colonies per plant were randomly taken and transferred to a fresh LB (Luria Broth) plate and the bacterial cells lysed at 95°C for 10 min. Plasmid inserts were amplified using the ITS1F and ITS4 primers under the following conditions: 94°C for 5 min (1 cycle); 94°C for 30 s, 55°C for 45 s, 72°C for 1 min (25 cycles); 72°C for 7 min (1 cycle).

Cloned ITS inserts were purified with Plasmid Purification Kit (QIAGEN) and sequenced with the same primer pair used for amplification. Dye sequencing was carried out on ABI 310 DNA Sequencer (Applied Biosystems, Carlsbad, California, USA).

Sequences were edited to remove vector sequence and to ensure correct orientation and assembled using the program Sequencher 4.1 for MacOsX from Genes Codes (Ann Arbor, Michigan). Sequence analysis was conducted with BLAST searches against the National Center for Biotechnology Information (NCBI) sequence database (GenBank, http://www.ncbi.nlm.nih.gov/BLAST/index.html) to determine closest sequence matches allowing taxonomic identification. DNA sequences were deposited in GenBank (Accession Numbers KT1222767–KT1222789).

Phylogenetic analysis was conducted using the software Mega v. 5.0 (Tamura et al., 2011). Sequences were aligned with Clustal X v. 2.0 (Larkin et al., 2007). Both a neighbour-joining tree and a maximum likelihood tree against selected database sequences were constructed using Kimura 2-parameter distances, with bootstrapping of 1000 replicates (Felsenstein, 1985). A *Geastrum* species (*G. schmidelii* Vittad.) was used as outgroup to root the tree, following Weiss et al. (2004).

2.4 Statistical analysis

In order to test the hypothesis that the two orchid species differ in terms of fungal communities, we calculated a community dissimilarity matrix using the Jaccard index on the individual plant-fungal taxon binary matrix and applied a PERMANOVA test to this matrix (McArdle & Anderson, 2001; Anderson, 2001; Legendre & Legendre, 1998; Oksanen et al., 2011). PERMANOVA is a non-parametric equivalent of MANOVA and solves all the issues that may affect the application of MANOVA to ecological matrices, especially binary ones. The main focus of the analysis was the factor "Orchid species": to avoid spurious effects due to sampling sites and spatial positions of samples, we tested for the main factor after statistically removing the effects of all other confounding factors. In any

case, factors such as “sampling site” did not have statistically significant effects on the fungal assemblage.

We compared fungal taxa richness between different orchid species and sites using rarefaction curves to “standardize” comparisons. We used various richness estimators (only Chao estimator is reported) to generate sample-based rarefaction curves of species richness and associated 95% CI.

All analyses were conducted with R version 3.0.2 (R Development Core Team, 2011) and the vegan package (Oksanen et al., 2011).

3 Results

3.1 Microscopical features of Cephalanthera mycorrhizal roots and fungal isolation

All *Cephalanthera* individuals collected in the three studied areas were dissected and examined. Microscopical observation clearly showed that both *C. damasonium* and *C. longifolia* roots were extensively colonized by fungi forming pelotons, dense intracellular hyphal coils, predominantly confined to the cortical cells and just marginally approaching the central stele (Fig. 1a, b, c). Fungal hyphae emanating from pelotons mostly appeared to be dark, septate and clamped, measuring 10-12 μm in diameter (Fig. 1d). Given the paucity of distinctive morphological characters, taxonomic delimitation of the orchid root associated fungi, based on microscopy, was extremely limited. These mycobionts were subsequently identified using molecular taxonomy.

Fungal *in vitro* isolation was in most cases unsuccessful or just yielded endophytic ascomycetous fungi, such as *Fusarium* strains, playing an ambiguous role in orchid-fungus interactions (Tondello et al., 2012; Pecoraro et al., 2015). However, mycelia that could be morphologically assigned to basidiomycetes by the presence of clamp connections were isolated from two *C. longifolia* plants, MR8 and CG1, respectively collected in “Monte Rotondo” and “Cornate di Gerfalco”. Ribosomal gene sequence analysis of these isolated fungi was further performed in order to assess their identity.

3.2 Molecular assessment of fungal diversity in C. damasonium and C. longifolia

Molecular approach revealed several fungal taxa colonizing *C. damasonium* and *C. longifolia* roots. Total DNA was extracted both from root tissues and from fungal cultures. PCR products with the ITS1F/ITS4 primer combination were obtained from 8 out of 11 analysed plants for each orchid

species. Fungal ITS sequencing following direct amplification of total orchid root DNA indicated that the two analysed orchid species were mostly associated with different fungal types (Table 1), even when sampled in sympatric populations, with only one exception represented by *Sebacina* species found both in *C. damasonium* (samples MC1 and MC4) and *C. longifolia* (sample CG4) from different areas. *Sebacina* sequences from the investigated orchids could be aligned with sequences from a variety of orchid and non-orchid plant species, as well as from fungal strains and fruitbodies. Both the neighbour-joining and the maximum likelihood trees showed that the *Sebacina*-like fungi identified in this work are phylogenetically close to other Sebacinaceae including *Sebacina incrustans* (Pers.) Tul. & C. Tul. and an uncultured ectomycorrhizal fungus associated with *Tuber magnatum* Picco in a natural truffle-ground from Italy (AJ879657), that formed a clade with the sequence from *C. longifolia* sample CG4, while Sebacinaceae sequences amplified from *C. damasonium* sample MC4 (clones a and b) and MC1 (clone e) respectively clustered with a sebacinoid fungus previously found in ectomycorrhizal root tip from *Tilia* sp. in Austria (AF509964) and with a mycobiont (AF440653) from roots of *Neottia nidus-avis* (L.) L.C. Rich. collected in France (100% bootstrap support, Figs. 2, 3).

As regards other fungal taxa collected from the roots of the studied orchids, results showed that *C. damasonium* associated with fungi belonging to Hymenogastraceae family, both in "Monte Cetona" and "Monte Rotondo" sampling areas (Table 1). Sequence from *C. damasonium* sample MC3 matched instead a sequence from *Ceratobasidium* sp. found in *Fragaria ananassa* Duch. The closest match for sample MC1 clone a was with *Cenococcum geophilum* Fr. from *F. sylvatica* in France, whereas clone d from sample MC4 shared 96% (over 881 bp) of similarity with an ascomycete detected on ectomycorrhizal root tips from an oak-dominated tropical montane cloud forest in Mexico. In "Monte Rotondo" Natural Reserve, the main fungal root associate of *C. damasonium* sample MR2 was a fungus with identity to Pezizomycetes found in *C. damasonium* roots in France.

By contrast, *C. longifolia* was found to be associated with *Tomentella* species in "Monte Rotondo" (Table 1). ITS sequencing of DNA extracted from basidiomycetous isolates obtained from *C. longifolia* showed that the studied orchid was associated with fungi belonging to Meruliaceae, *Bjerkandera adusta* (Willd.) P. Karst. from sample MR8 and *Phlebia acerina* Peck from sample CG1, in the two investigated areas.

Ascomycetes such as *Davidiella macrospora* (Kleb.) Crous & U. Braun, *Exophiala salmonis* J.W. Carmich., *Neonectria radicicola* (Gerlach & L. Nilsson) Mantiri & Samuels, *Tetracladium* sp. and *Fusarium* sp. were sporadically found in the two analysed orchid species.

Statistical results

There was not statistical difference between the fungal communities associated with the two orchid species. This negative result is due to the fact that each and every individual plant possess one or two fungal associates but the identity of the fungi changes from plant to plant, and so between the two orchid species: there is not a set of fungi uniquely associated with an orchid species. Overall, this result implies a thorough fungal compositional turnover from plant to plant and high number of fungal taxa retrieved on a collection of plants belonging to the same orchid species, with no difference in richness between orchid species. For example, the estimated mean (±95% CI) number of fungal taxa was 26 ± 14 for *C. damasonium* and 30 ± 22 for *C. longifolia*.

4 Discussion

The accurate analysis of fungal diversity associated with *C. damasonium* and *C. longifolia* performed in the present work, using fungal isolation, microscopy, and DNA sequencing, uncovered a range of fungi in the roots of the investigated orchids.

The first evidence of the establishment of orchid-fungus associations in the studied orchid species was provided by microscopic observations, showing abundant pelotons in the sampled root cells, which represent the main morphological aspect of orchid mycorrhiza (Rasmussen, 1995; Kristiansen et al., 2001).

In spite of the poor success in the isolation of fungal associates from the analysed *Cephalanthera* plant roots, culture-dependent methods complemented PCR-based approach, as the basidiomycetes isolated from *C. longifolia* yielded ITS sequences that were not amplified using direct total orchid root DNA amplification. These fungal isolates from samples MR8 and CG1 respectively showed close identity to *B. adusta* and *P. acerina*, two species of wood-decaying fungi, in the Meruliaceae family, that colonize both hardwood and conifer wood, and are capable of degrading a variety of substrates by secreting specific enzymes (Nakasone & Sytsma, 1993; Romero et al., 2007). Wood-inhabiting fungal species have been previously identified in orchid mycorrhizal associations.

298 *Erythromyces crocicreas* (Berk. & Broome) Hjortstam & Ryvarden has been found to support seed
299 germination and to be effective for further development of the orchid *Galeola altissima* (Bl.) Reichb. f.
300 (Umata, 1995), while *Armillaria mellea* (Vahl) P. Kumm. has been shown to associate with *Gastrodia*
301 *elata* Blume (Kusano, 1911; Kikuchi et al., 2008). *Resinicium* sp., that is a member of Meruliaceae, has
302 been identified by Martos et al., (2009) in *Gastrodia similis* Bosser mycorrhizal roots, using molecular
303 methods. Our findings of Meruliaceae fungi in the roots of *C. longifolia* could support the hypothesis
304 of a trophic relationship between the studied orchid and wood-inhabiting fungal taxa, although further
305 analyses are needed to verify this hypothesis.
306 A range of fungi was identified through fungal ITS sequencing from amplified total orchid
307 root DNA. Neither *C. damasonium* nor *C. longifolia* were associated with a dominant fungal taxon,
308 thus showing a low level of mycorrhizal specificity that is consistent with their mixotrophic life style
309 (Selosse et al., 2004; Dearnaley et al., 2012). To date, specificity in associations between
310 *Cephalanthera* species and fungal symbionts has been controversial. Previous studies have been mostly
311 based on a very limited number of *Cephalanthera* plant samples collected in a single site. As a
312 consequence, results obtained from these studies could not provide a clear picture of fungal diversity
313 associated with *C. damasonium* and *C. longifolia*, and therefore, exhaustive information on their
314 mycorrhizal specificity has been hardly available. Bidartondo et al. (2004) and Liebel et al. (2010) have
315 found low degree of mycorrhizal specificity in *C. damasonium* and *C. longifolia* respectively, the
316 fungal associates being *Cortinarius*, *Hymenogaster*, *Inocybe*, *Thelephora*, and *Tomentella* in *C.*
317 *damasonium*, while *C. longifolia* has been found to associate with *Hebeloma*, *Russula*, and *Tomentella*.
318 On the contrary, Abadie et al. (2006) have shown a very specific association between *C. longifolia* and
319 Thelephoraceae considering that all ITS, in a typing of fungal ITS on 60 orchid root pieces (from 7
320 plants) harbouring pelotons, were from thelephoroid fungi, with the exception of two root pieces
321 exhibiting ITS from a Sebacinaceae and an ascomycete belonging to *Wilcoxina*. Interesting information
322 about mycorrhizal associations in *C. damasonium* has been provided by Julou et al. (2005). These
323 authors have amplified a number of asco- and basidiomycetes sequences from 4 *C. damasonium*
324 samples collected from a single population in France, using ITS sequencing. Among these sequenced
325 fungi, Thelephoraceae, Cortinariaceae (including *Hymenogaster*), and Pezizomycetes (including
326 Helotiales, Pezizales, *Tuber*, *Phialophora*, and *Leptodontidium*) were over-represented. One *Sebacina*
327 and one *Ceratobasidium* sequences were also amplified from the same orchid plants. Julou and co-

328 authors, in the analysis of data collected during their investigation, have proposed a quite specific
329 mycorrhizal association between *C. damasonium*, Thelephoraceae and Cortinariaceae, but at the same
330 time, they have not excluded a symbiotic role for the other fungal taxa identified from the studied
331 orchid roots. The only one previous work, in which *C. damasonium* and *C. longifolia* have been
332 investigated together, for their mycorrhizal associations, has reported on a high degree of specificity in
333 the relationship between the two investigated orchids and Thelephoraceae fungi during the seedling
334 stage, while mycorrhizal specificity was lower in different plant life stages (Bidartondo & Read, 2008).
335 Indeed, these authors have found seedlings of both *Cephalanthera* species to associate with only a
336 subset of the thelephoroid fungi capable of stimulating seed germination, and to lack completely
337 Cortinariaceae and Sebacinaceae that have been collected from germinating seeds and mature plants.
338 The present study, based on the analysis of 22 orchid samples from three geographically distinct
339 protected areas to allow statistical validation, revealed at least 11 fungal taxa colonizing *C.*
340 *damasonium* roots, while about 9 endophytic fungal types were found to associate with *C. longifolia*
341 (Table 1). Such a large diversity of fungal associates strongly suggests that both *C. damasonium* and *C.*
342 *longifolia* are generalist in their mycorrhizal associations. Statistical analyses clearly show distinct
343 fungal communities associated with each and every orchid plant individual, irrespective of the orchid
344 species and the site of origin.

345 We mainly found different fungal associates in the two investigated orchids (Table 1).
346 *Sebacina* represents the only putative mycorrhizal fungus that was associated with both *C. damasonium*
347 (two plants on “Monte Cetona”) and *C. longifolia* (one plant on “Cornate di Gerfalco”). Phylogenetic
348 analysis suggests that the sequences of sebacinoid fungi amplified from the studied orchids are
349 sufficiently different to include at least three related species (Figs. 2, 3). *C. longifolia* mycobiont has
350 close affinity with *S. incrustans* previously found in symbiotic relationship with the achlorophyllous
351 orchid *N. nidus-avis* (Selosse et al., 2002) and with Sebacinaceae playing a symbiotic role in
352 unidentified morphotypes of ectomycorrhizal tips collected in a truffle-ground by Murat et al. (2005).
353 *C. damasonium* sebacinoid associates are instead phylogenetically close to Sebacinaceae that have been
354 previously found to establish mycorrhizal associations with orchid (Selosse et al., 2002) and non-orchid
355 plants (Urban et al., 2003). Sebacinoid basidiomycetes show a remarkable diversity of mycorrhizal
356 types (Weiss et al., 2004). Moreover, Sebacinoid mycobionts can support the myco-heterotrophic
357 growth of achlorophyllous orchids (McKendrick et al., 2002; Selosse et al., 2002; Taylor et al., 2003)

as well as they can play a functional role in association with chlorophyllous orchid species (Bonnardeaux et al., 2007; Wright et al., 2010). Considering the puzzling variety of symbiotic plant-fungus associations in which Sebacinaceae are involved, including orchid mycorrhizas, we speculate that sebacinoid fungi found in the present study from the roots of *C. damasonium* and *C. longifolia*, may play a trophic role in their relationship with the analysed orchids. Much further work is required to provide an integrated view of *Sebacina-Cephalanthera* interactions based on morphological, molecular, and physiological data.

Together with *Sebacina*, another fungal taxon belonging to the anamorphic form genus *Rhizoctonia* was associated with only one of the two analysed orchids, the best match for the sequence amplified from *C. damasonium* sample MC3 being with *Ceratobasidium* sp. collected by Sharon et al. (2007) from *F. ananassa* in Israel. Ceratobasidioid fungi have been found in symbiotic associations with terrestrial orchids from both forests and meadows, as well as epiphytic orchids (Shefferson et al., 2005; Otero et al., 2005; Otero et al., 2007, 2011; Irwin et al., 2007; Yagame et al., 2008, 2012; Girlanda et al., 2011; Jacquemyn et al., 2011a, b; Pecoraro et al., 2012a; Tondello et al., 2012). Moreover, the trophic relationship between a *Ceratobasidium* species *C. cornigerum* (Bourdot) D.P. Rogers and the green forest orchid *Goodyera repens* (L.) R. Br. represents the first known example of mutualistic mycorrhiza in orchids (Cameron et al., 2006). The second best match for the *Ceratobasidium* sequence that was amplified from *C. damasonium* in the present work is with *C. cornigerum* (isolate XSD-44, Jiang et al., unpublished, GenBank record EU273525). We propose the role of symbiont for this *Ceratobasidium* taxon associated with the roots of *C. damasonium*, although the confirmation of its exact physiological role would require detailed analyses.

Ectomycorrhizal (ECM) fungi were also found to colonize the roots of the two investigated *Cephalanthera* species. Hymenogastraceae were collected from 37,5 % of *C. damasonium* analysed plants, being the only fungal partners associated with the studied orchid in both of the two geographical distinct sampling areas ("Monte Cetona" and "Monte Rotondo", see Table 1). All the Hymenogastraceae sequences amplified in the present work had the same BLAST search closest match with a *C. damasonium* mycorrhizal symbiont (GenBank accession code AY634136) collected by Bidartondo et al. (2004) from orchid root samples, in a German forest site. Identification of *C. damasonium* mycobiont as a member of Hymenogastraceae family is supported by the high similarity that the sequences collected in the present study also showed, as their second best match, with

388 sequences amplified from *Hymenogaster bulliardii* Vittad. (Peintner et al., 2001) and *H. citrinus*
389 Vittad. (Brock et al., 2009) specimens deposited in herbaria. ECM thelephoroid basidiomycetes
390 belonging to *Tomentella* were instead associated with *C. longifolia* in "Monte Rotondo" Natural
391 Reserve. Tomentelloid fungi found in the roots of the studied orchid shared similarity with *Tomentella*
392 species involved in ectomycorrhizal associations with several tree species growing in boreal forests
393 (Tedersoo et al., 2003; Kjøller, 2006; Bidartondo & Read, 2008). In particular, *Tomentella* taxa
394 detected in tree ectomycorrhizas in England by Bidartondo & Read (2008) have been shown to support
395 both *C. damasonium* and *C. longifolia* seed germination, seedling development and adult plant growth.
396 Symbiotic associations with thelephoroid fungi have previously been reported in several
397 *Cephalanthera* species including *C. austiniae* (Taylor & Bruns, 1997), *C. damasonium* (Bidartondo et
398 al., 2004; Julou et al., 2005; Bidartondo & Read, 2008), *C. erecta* Blume (Matsuda et al., 2009), *C.*
399 *falcata* Blume (Yamato & Iwase, 2008; Matsuda et al., 2009), *C. longifolia* (Abadie et al., 2006;
400 Bidartondo & Read, 2008; Liebel et al., 2010), and *C. rubra* Rich. (Bidartondo et al., 2004). Yagame &
401 Yamato (2012) demonstrated the establishment of tripartite symbioses between the mycoheterothrophic
402 orchid *C. falcata*, Thelephoraceae fungi, and *Quercus serrata* Murray (Fagaceae), in culture condition.
403 The simultaneous association with rhizoctonia-forming fungi and ECM fungal groups is a
404 noteworthy aspect of mycorrhizal diversity in the analysed *Cephalanthera* species. This feature
405 suggests that *C. damasonium* and *C. longifolia* represent an intermediate step in the evolution from
406 fully photosynthetic orchids, mostly associated with *Rhizoctonia*-like fungi, to fully mycoheterotrophic
407 orchids, often found in symbiosis with ECM fungal associates (Bidartondo et al., 2004; Motomura et
408 al., 2010; Dearnaley et al., 2012). It has been previously suggested by Taylor and Bruns (1997) that the
409 achlorophyllous mycoheterotrophic *Cephalanthera* species *C. austinae* evolved from autotrophic
410 ancestors by switching from *Rhizoctonia* to thelephoroid fungal partners, as a probable adaptation for
411 colonizing dark, understory habitats.
412 Other fungal ITS sequences sporadically collected from the roots of the studied orchid species
413 deserve to be discussed. Several mycorrhizal fungi previously found in a variety of environmental
414 sources, such as *C. geophilum* from *F. sylvatica* ECM root tips (Buée et al., 2005), an ectomycorrhizal
415 ascomycete from root tips in a tropical cloud Mexican forest (Morris et al., 2008), and pezizomycetes
416 from orchid roots (Julou et al., 2005) were the best matches for sequences detected in *C. damasonium*.
417 We cannot exclude that these fungal associates may be involved in a trophic relationship with the

418 analysed orchid. They could represent occasional symbionts for mycorrhizal associations that the
419 investigated orchid explores, looking for the best partners available from the fungal community that
420 characterizes a particular habitat.

421 A fungal taxon with 99% identity to *Tetracladium* sp. found in orchid roots (Abadie et al.,
422 2006) and mycorrhizal roots of conifer seedlings (Menkis et al., 2005) was identified in *C. longifolia*.
423 The genus *Tetracladium* is a member of the so-called Ingoldian fungi, asexual microfungi commonly
424 occurring on dead plant material, in running freshwater (Bärlocher, 1992). These aquatic hyphomycetes
425 have been recently found as endophytes in several plant tissues, including orchid roots (Abadie et al.,
426 2006; Vendramin et al., 2010). Selosse et al. (2008) proposed that some Ingoldian fungi spend part of
427 their life in plants and use water for dispersion. These authors suggested to investigate the impact of
428 Ingoldian fungi on plant species, during their endophytic life stage, in order to test their protective
429 effects on hosts. Other putative endophytic fungi, with a diverse and mostly unknown ecology, were
430 detected in the studied orchids. Among them, *E. salmonis* was found in the roots of *C. damasonium*
431 sample MR2, showing similarity with a fungal strain collected from *Salmo clarkii* (Untereiner &
432 Naveau, 1999). *Exophiala salmonis* has been reported from the roots of several orchid species, such as
433 *Orchis pauciflora* Tenore (Pecoraro et al., 2012b) and *Himantoglossum adriaticum* H. Baumann
434 (Pecoraro et al., 2013) and has been also detected in the roots of *C. damasonium* adult plants by Julou
435 et al. (2005). Such findings of orchid fungal associates that show a large ecological plasticity and are
436 capable to play different roles in their interactions with several host organisms deserve further
437 attention. More studies on their endophytic phase in orchid hosts are necessary to clarify their real
438 function in colonized plants. These intriguing fungi could represent "unexpected" orchid symbionts
439 with some important nutritional roles. A better understanding of the real diversity of orchid
440 mycorrhizal fungi is a fundamental starting point to support any consideration on orchid-fungus
441 specificity.
442
443 **Acknowledgements**

444 The experimental work for this study has been performed within the doctoral research project
445 of L. Pecoraro (PhD School in Applied Environmental Sciences and Technologies, University of
446 Siena). L. Pecoraro acknowledges CAS 153211KYSB20160029 for supporting his research at Chinese
447 Academy of Sciences.

448

449 **References**

450 Abadie JC, Püttsepp Ü, Gebauer G, Faccio A, Bonfante P, Selosse MA. 2006. *Cephalanthera*
451 *longifolia* (Neottieae, Orchidaceae) is mixotrophic: a comparative study between green and
452 nonphotosynthetic individuals. *Canadian Journal of Botany* 84: 1462-1477.

453 Anderson MJ. 2001. A new method for non-parametric multivariate analysis of variance. *Austral*
454 *Ecology* 26: 32–46.

455 Bärlocher F. 1992. *The ecology of aquatic hyphomycetes*. Berlin: Springer-Verlag.

456 Bateman RM, Hollingsworth PM, Squirrell J, Hollingsworth ML. 2004. Tribe Neottieae:
457 phylogenetics. In: Pridgeon AM, Cribb PL, Chase MW, Rasmussen FN eds. *Genera*
458 *Orchidacearum (4): Epidendroideae (part 1)*. Oxford: Oxford University Press. 487-495.

459 Bidartondo MI, Burghardt B, Gebauer G, Bruns TD, Read DJ. 2004. Changing partners in the dark:
460 isotopic and molecular evidence of ectomycorrhizal liaisons between forest orchids and trees.
461 *Proceedings of the Royal Society of London B* 271: 1799–1806.

462 Bidartondo MI, Read DJ. 2008. Fungal specificity bottlenecks during orchid germination and
463 development. *Molecular Ecology* 17: 3707-3716.

464 Bonnardeaux Y, Brunndrett M, Batty A, Dixon K, Koch J, Sivasithamparam K. 2007. Diversity of
465 mycorrhizal fungi of terrestrial orchids: compatibility webs, brief encounters, lasting relationships
466 and alien invasions. *Mycological Research* 111: 51-61.

467 Brock PM, Döring H, Bidartondo MI. 2009. How to know unknown fungi: the role of a herbarium.
468 *New Phytologist* 181: 719-724.

469 Buée M, Vairelles D, Garbaye J. 2005. Year-round monitoring of diversity and potential metabolic
470 activity of the ectomycorrhizal community in a beech (*Fagus sylvatica*) forest subjected to two
471 thinning regimes. *Mycorrhiza* 15: 235-245.

472 Cameron DD, Leake JR, Read DJ. 2006. Mutualistic mycorrhiza in orchids: evidence from plant-
473 fungus carbon and nitrogen transfers in the green-leaved terrestrial orchid *Goodyera repens*. *New*
474 *Phytologist* 171: 405-416.

475 Colemann RA. 1995. *The wild orchids of California*. Cornell Univ. Press.

476 Dearnaley JDW, Bougoure JJ. 2010. Isotopic and molecular evidence for saprotrophic Marasmiaceae
477 mycobionts in rhizomes of *Gastrodia sesamoides*. *Fungal Ecology* 3: 288-294.

478 Dearnaley JDW, Martos F, Selosse MA. 2012. Orchid mycorrhizas: molecular ecology, physiology,
479 evolution, and conservation aspects. In: Hock B ed. *Fungal associations, 2nd edn*. Berlin: Springer-
480 Verlag. 207-230.
481 Delforge P. 2006. *Orchids of Europe, North Africa and the Middle East*. Oregon: Timber press Inc.
482 Doyle JJ, Doyle JL. 1990. Isolation of plant DNA from fresh tissues. *Focus* 12: 13–15.
483 Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39:
484 783-791.
485 Gale SW, Yamazaki J, Hutchings MJ, Yukawa T, Miyoshi K. 2010. Constraints on establishment in an
486 endangered terrestrial orchid: a comparative study of in vitro and in situ seed germinability and
487 seedling development in *Nervilia nipponica*. *Botanical Journal of the Linnean Society* 163: 166-
488 180.
489 Gardes M, Bruns TD. 1993. ITS primers with enhanced specificity for basidiomycetes - applications to
490 the identification of mycorrhizae and rusts. *Molecular Ecology* 2: 113–118.
491 Gebauer G, Meyer M. 2003. ^{15}N and ^{13}C natural abundance of autotrophic and mycoheterotrophic
492 orchids provides insight into nitrogen and carbon gain from fungal association. *New Phytologist*
493 160: 209-223.
494 Girlanda M, Selosse MA, Cafasso D, Brilli F, Delfine S, Fabbian R, Ghignone S, Pinelli P, Segreto R,
495 Loreto F, Cozzolino S, Perotto S. 2006. Inefficient photosynthesis in the Mediterranean orchid
496 *Limodorum abortivum* is mirrored by specific association to ectomycorrhizal Russulaceae.
497 *Molecular Ecology* 15: 491-504.
498 Girlanda M, Segreto R, Cafasso D, Liebel HT, Rodda M, Ercole E, Cozzolino S, Gebauer G, Perotto S.
499 2011. Photosynthetic Mediterranean meadow orchids feature partial mycoheterotrophy and specific
500 mycorrhizal associations. *American Journal of Botany* 98: 1148-1163.
501 Illyés Z, Ouanphanivanh N, Rudnóy S, Orczán ÁK, Bratek Z. 2010. The most recent results on orchid
502 mycorrhizal fungi in Hungary. *Acta Biologica Hungarica* 61: 68-76.
503 Irwin MJ, Bougoure JJ, Dearnaley JDW. 2007. *Pterostylis nutans* (Orchidaceae) has a specific
504 association with two *Ceratobasidium* root associated fungi across its range in Eastern Australia.
505 *Mycoscience* 48: 231-239.

506 Jacquemyn H, Honnay O, Cammue BPA, Brys R, Lievens B. 2010. Low specificity and nested subset
507 structure characterize mycorrhizal associations in five closely related species of the genus *Orchis*.
508 *Molecular Ecology* 19: 4086-4095.
509 Jacquemyn H, Brys R, Cammue BPA, Honnay O, Lievens B. 2011a. Mycorrhizal associations and
510 reproductive isolation in three closely related *Orchis* species. *Annals of Botany* 107: 347-356.
511 Jacquemyn H, Merckx V, Brys R, Tyteca D, Cammue BPA, Honnay O, Lievens B. 2011b. Analysis of
512 network architecture reveals phylogenetic constraints on mycorrhizal specificity in the genus
513 *Orchis* (Orchidaceae). *New Phytologist* 192: 518–528.
514 Julou T, Burghardt B, Gebauer G, Berveiller D, Damesin C, Selosse MA. 2005. Mixotrophy in
515 Orchids: insights from a comparative study of green individuals and nonphotosynthetic individuals
516 of *Cephalantera damasonium*. *New Phytologist* 166: 639-653.
517 Kikuchi G, Higuchi M, Morota T, Nagasawa E, Suzuki A. 2008. Fungal symbiont and cultivation test
518 of *Gastrodia elata* Blume (Orchidaceae). *Jpn J Bot* 83: 88-95.
519 Kjøller R. 2006. Disproportionate abundance between ectomycorrhizal root tips and their associated
520 mycelia. *FEMS Microbiology Ecology* 58: 214-224.
521 Kristiansen KA, Taylor DL, Kjøller R, Rasmussen HN, Rosendahl S. 2001. Identification of
522 mycorrhizal fungi from single pelotons of *Dactylorhiza majalis* (Orchidaceae) using SSCP and
523 mitochondrial ribosomal LsDNA sequences. *Molecular Ecology* 10: 2089-2093.
524 Kusano S. 1911. *Gastrodia elata* and its symbiotic association with *Armillaria mellea*. *J Coll Agric Jpn*
525 9: 1-73.
526 Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, et al.
527 2007. Clustal W and Clustal X version 2.0. *Bioinformatics* 23: 2947-2948.
528 Leake JR. 1994. The biology of mycoheterotrophic (‘saprophytic’) plants. Tansley Review No. 69.
529 *New Phytologist* 127: 171-216.
530 Legendre P, Legendre L. 1998. *Numerical ecology, 2nd edn*. Amsterdam: Elsevier Science.
531 Liebel HT, Bidartondo MI, Preiss K, Segreto R, Stöckel M, Rodda M, Gebauer G. 2010. C and N
532 stable isotope signatures reveal constraints to nutritional modes in orchids from the Mediterranean
533 and Macaronesia. *American Journal of Botany* 97: 903-912.

534 Martos F, Dulormne M, Pailler T, Bonfante P, Faccio A, Fournel J, Dubois MP, Selosse MA. 2009.
535 Indipendent recruitment of saprotrophic fungi as mycorrhizal partners by tropical achlorophyllous
536 orchids. *New Phytologist* 184: 668-681.
537 Matsuda Y, Amiya A, Ito S. 2009. Colonization patterns of mycorrhizal fungi associated with two rare
538 orchids, *Cephalanthera falcata* and *C. erecta*. *Ecological Research* 24: 1023–1031.
539 McArdle BH, Anderson MJ. 2001. Fitting multivariate models to community data: A comment on
540 distance-based redundancy analysis. *Ecology* 82: 290–297.
541 McCormick MK, Jacquemyn H. 2014. What constrains the distribution of orchid population? *New*
542 *Phytologist* 202: 392-400.
543 McKendrick SL, Leake JR, Taylor DL, Read DJ. 2002. Symbiotic germination and development of the
544 myco-heterotrophic orchid *Neottia nidus-avis* in nature and its requirement for locally distributed
545 *Sebacina* spp. *New Phytologist* 154: 233-247.
546 Menkis A, Vasiliauskas R, Taylor AFS, Stenlid J, Finlay R. 2005. Fungal communities in mycorrhizal
547 roots of conifer seedlings in forest nurseries under different cultivation systems, assessed by
548 morphotyping, direct sequencing and mycelial isolation. *Mycorrhiza* 16: 33-41.
549 Merckx V, Bidartondo MI, Hynson NA. 2009. Myco-heterotrophy: when fungi host plants. *Annals of*
550 *Botany* 104: 1255-1261.
551 Morris MH, Pérez-Pérez MA, Smith ME, Bledsoe CS. 2008. Multiple species of ectomycorrhizal fungi
552 are frequently detected on individual oak root tips in a tropical cloud forest. *Mycorrhiza* 18: 375-
553 383.
554 Motomura H, Selosse MA, Martos F, Kagawa A, Yukawa T. 2010. Mycoheterotrophy evolved from
555 mixotrophic ancestors: evidence in *Cymbidium* (Orchidaceae). *Annals of Botany* 106: 573-581.
556 Murat C, Vizzini A, Bonfante P, Mello A. 2005. Morphological and molecular typing of the below-
557 ground fungal community in a natural *Tuber magnatum* truffle-ground. *FEMS Micobiology Letters*
558 245: 307-313.
559 Nakasone KK, Sytsma KJ. 1993. Biosystematic studies on *Phlebia acerina*, *P. rufa*, and *P. radiata* in
560 North America. *Mycologia* 85: 996-1016.
561 Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB, Simpson GL, Solymos P,
562 Stevens MHH, Wagner H. 2011. Vegan: community ecology package. R package version 2.0-2.

563 Otero JT, Bayman P, Ackerman JD. 2005. Variation in mycorrhizal performance in the epiphytic
564 orchid *Tolumnia variegata* in vitro: the potential for natural selection. *Evolutionary Ecology* 19: 29-
565 43.
566 Otero JT, Flanagan NS, Herre EA, Ackerman JD, Bayman P. 2007. Widespread mycorrhizal specificity
567 correlates to mycorrhizal function in the neotropical, epiphytic orchid *Ionopsis utricularioides*
568 (Orchidaceae). *American Journal of Botany* 94: 1944-1950.
569 Otero JT, Thrall PH, Clements M, Burdon JJ, Miller JT. 2011. Codiversification of orchids
570 (Pterostylidinae) and their associated mycorrhizal fungi. *Australian Journal of Botany* 59: 480–497.
571 Pecoraro L, Girlanda M, Kull T, Perini C, Perotto S. 2012a. Analysis of fungal diversity in *Orchis*
572 *tridentata* Scopoli. *Central European Journal of Biology* 7: 850–857.
573 Pecoraro L, Girlanda M, Kull T, Perini C, Perotto S. 2012b. Molecular identification of root fungal
574 associates in *Orchis pauciflora* Tenore. *Plant Biosystems* 146(4): 985–991.
575 Pecoraro L, Girlanda M, Kull T, Perini C, Perotto S. 2013. Fungi from the roots of the terrestrial
576 photosynthetic orchid *Himantoglossum adriaticum*. *Plant Ecology and Evolution* 146(2): 145–152.
577 Pecoraro L, Girlanda M, Liu ZJ, Huang LQ, Perotto S. 2015. Molecular analysis of fungi associated
578 with the Mediterranean orchid *Ophrys bertolonii* Mor. *Annals of Microbiology*. 65: 2001-2007.
579 Peintner U, Bougher NL, Castellano MA, Moncalvo JM, Moser MM, Trappe JM, Vilgalys R. 2001.
580 Multiple origins of the sequestrate fungi related to *Cortinarius* (Cortinariaceae). *American Journal*
581 *of Botany* 88(12): 2168-2179.
582 Phillips RD, Brown AP, Dixon KW, Hopper SD. 2011. Orchid biogeography and factors associated
583 with rarity in a biodiversity hotspot, the Southwest Australian Floristic Region. *Journal of*
584 *Biogeography* 38: 487-501.
585 Preiss K, Adam IKU, Gebauer G. 2010. Irradiance governs exploitation of fungi: fine-tuning of carbon
586 gain by two partially myco-heterotrophic orchids. *Proceedings of the Royal Society of London B*
587 277: 1333-1336.
588 R Development Core Team. 2011. *R: a language and environment for statistical computing*. Vienna: R
589 Foundation for Statistical Computing.
590 Rasmussen HN. 1995. *Terrestrial orchids: From seed to mycotrophic plant*. Cambridge: Cambridge
591 University press.

592 Rasmussen HN, Rasmussen FN. 2009. Orchid mycorrhiza: implications of a mycophagous life style.
593 *Oikos* 118: 334-345.
594 Romero E, Speranza M, García-Guinea J, Martínez AT, Martínez MJ. 2007. An anamorph of the
595 white-rot fungus *Bjerkandera adusta* capable of colonizing and degrading compact disc
596 components. *FEMS Microbiology Letters* 275: 122-9.
597 Rossi W. 2002. *Orchidee d'Italia*. Quad. Cons. Natura, 15, Min. Ambiente-Ist. Naz. Fauna Selvatica.
598 Roy M, Watthana S, Stier A, Richard F, Vessabutr S, Selosse MA. 2009. Two mycoheterotrophic
599 orchids from Thailand tropical dipterocarpacean forests associate with a broad diversity of
600 ectomycorrhizal fungi. *BMC Biology* 7: 51.
601 Selosse MA, Weiß M, Jany JL, Tillier A. 2002. Communities and populations of sebacinoid
602 basidiomycetes associated with the achlorophyllous orchid *Neottia nidus-avis* (L.) L.C.M. Rich. and
603 neighbouring tree ectomycorrhizae. *Molecular Ecology* 11: 1831-1844.
604 Selosse MA, Faccio A, Scappaticci P, Bonfante P. 2004. Chlorophyllous and achlorophyllous
605 specimens of *Epipactis microphylla* (Neottieae, Orchidaceae) are associated with ectomycorrhizal
606 septomycetes, including truffles. *Microbiological Ecology* 47: 416-426.
607 Selosse MA, Vohník M, Chauvet E. 2008. Out of the rivers: Are some aquatic hyphomycetes plant
608 endophytes? *New Phytologist* 178: 3-7.
609 Selosse MA, Roy M. 2009. Green plants that feed on fungi: facts and questions about mixotrophy.
610 *Trends in Plant Science* 14: 64-70.
611 Sharon M, Freeman S, Kuninaga S, Sneh B. 2007. Genetic diversity, anamostosis groups and virulence
612 of *Rhizoctonia* spp. from strawberry. *European Journal of Plant Pathology* 117: 247-265.
613 Shefferson RP, Weiss M, Kull T, Taylor DL. 2005. High specificity generally characterizes
614 mycorrhizal association in rare lady's slipper orchids, genus *Cypripedium*. *Molecular Ecology* 14:
615 613–626.
616 Smith SE. 1967. Carbohydrate translocation in orchid mycorrhizas. *New Phytologist* 66: 371-378.
617 Smith SE, Read DJ. 1997. *Mycorrhizal Symbiosis, 2nd ed*. San Diego: Academic Press.
618 Smith SE, Read DJ. 2008. *Mycorrhizal Symbiosis, 3rd ed*. San Diego: Academic Press.
619 Swarts ND, Dixon KW. 2009. Terrestrial orchid conservation in the age of extinction. *Annals of Botany*
620 104: 543-556.

621 Swarts ND, Sinclair EA, Francis A, Dixon KW. 2010. Ecological specialization in mycorrhizal
622 symbiosis leads to rarity in an endangered orchid. *Molecular Ecology* 19: 3226-3242.
623 Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA5: Molecular
624 Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum
625 Parsimony Methods. *Molecular Biology and Evolution* 28: 2731-2739.
626 Taylor DL, Bruns TD. 1997. Indipendent, specialized invasions of ectomycorrhizal mutualism by two
627 nonphotosynthetic orchids. *Proceedings of the National Academy of Sciences of the USA* 94: 4510-
628 4515.
629 Taylor DL, Bruns TD, Leake JR, Read DJ. 2002. Mycorrhizal specificity and function in myco-
630 heterotrophic plants. In: Van der Heijden MGA, Sanders I eds. *Mycorrhizal Ecology*. *Ecological*
631 *Studies* 157: 375-413.
632 Taylor DL, Bruns TD, Szaro TM, Hodges SA. 2003. Divergence in mycorrhizal specialization within
633 *Hexalectris spicata* (Orchidaceae), a nonphotosynthetic desert orchid. *American Journal of Botany*
634 90: 1168-1179.
635 Tedersoo L, Kõljalg U, Hallenberg N, Larsson KH. 2003. Fine scale distribution of ectomycorrhizal
636 fungi and roots across substrates layers including coarse woody debris in a mixed forest. *New*
637 *Phytologist* 159: 153-165.
638 Tondello A, Vendramin E, Villani M, Baldan B, Squartini A. 2012. Fungi associated with the southern
639 Eurasian orchid *Spiranthes spiralis* (L.) Chevall. *Fungal Biology* 116: 543-549.
640 Umata H. 1995. Seed germination of *Galeola altissima*, an achlorophyllous orchid with
641 aphyllophorales fungi. *Mycoscience* 36: 369-372.
642 Urban A, Weiß M, Bauer R. 2003. Ectomycorrhizas involving sebacinoid mycobionts. *Mycological*
643 *Research* 107: 3-14.
644 Untereiner WA, Naveau FA. 1999. Molecular systematics of the Herpotrichiellaceae with an
645 assessment of the phylogenetic positions of *Exophiala dermatitidis* and *Phialophora americana*.
646 *Mycologia* 91: 67–83.
647 Vendramin E, Gastaldo A, Tondello A, Baldan B, Villani M, Squartini A. 2010. Identification of two
648 fungal endophytes associated with the endangered orchid *Orchis militaris* L. *Journal of*
649 *Microbiology and Biotechnology* 20: 630–636.

Weiss M, Selosse MA, Rexer KH, Urban A, Oberwinkler F. 2004. Sebacinales: a hitherto overlooked cosm of heterobasidiomycetes with a broad mycorrhizal potential. *Mycological Research* 108(9): 1003-1010.

Wright MM, Cross R, Cousens RD, May TW, McLean CB. 2010. Taxonomic and functional characterization of fungi from the *Sebacina vermifera* complex from common and rare orchids in the genus *Caladenia*. *Mycorrhiza* 20: 375-390.

Yagame T, Yamato M. 2012. Mycoheterotrophic growth of *Cephalanthera falcata* (Orchidaceae) in tripartite symbioses with Thelephoraceae fungi and *Quercus serrata* (Fagaceae) in pot culture. *Journal of Plant Research*. doi: 10.1007/s10265-012-0521-7.

Yagame T, Yamato M, Suzuki A, Iwase K. 2008. Ceratobasidiaceae mycorrhizal fungi isolated from nonphotosynthetic orchid *Chamaegastrodia sikokiana*. *Mycorrhiza* 18: 97–101.

Yagame T, Orihara T, Selosse MA, Yamato M, Iwase K. 2012. Mixotrophy of *Platanthera minor*, an orchid associated with ectomycorrhiza-forming Ceratobasidiaceae fungi. *New Phytologist* 193: 178–187.

Yamato M, Iwase K. 2008. Introduction of asymbiotically propagated seedlings of *Cephalanthera falcata* (Orchidaceae) into natural habitat and investigation of colonized mycorrhizal fungi. *Ecological Research* 23: 329–337.

Table 1. Fungal diversity molecularly detected in the analysed orchids. BLAST search closest matches of fungal ITS-DNA sequences amplified from C. damasonium and C. longifolia roots collected in "Monte Cetona" (samples MC1-MC4), "Monte Rotondo" (samples MR1-MR8), and "Cornate di Gerfalco" (samples CG1-CG4). Sample GenBank accession codes, accession codes for the closest GenBank matches, sequence identity, and overlap of each match are reported.

Orchid species	Sample	Clone	Site	GenBank code	Best BLAST match(es)	Accession code	Overlap lenght	% match
C. damasonium	MC1	a	B	KT122776	*Cenococcum geophilum* (from ectom.)	AY299214	610	93%
		c	B	KT122777	*Cryptococcus carnescens*	AB105438	854	98%
		e	B	KT122778	*Sebacina* (from *N. nidus-avis*)	AF440653	1002	96%
					Sebacina aff. *epigaea*	AF490393	841	91%
	MC2	c	B	KT122779	*Hymenogastraceae* (from *C. damasonium*)	AY634136	1146	98%
					Hymenogaster bulliardii	AF325641	1009	95%
	MC3	c	A	KT122780	*Ceratobasidium* sp. (from *F. ananassa*)	DQ102416	765	87%
					Ceratobasidium cornigerum	EU273525	763	87%
	MC4	a	A	KT122781	*Sebacinaceae* (from *E. helleborine*)	AY452676	1003	96%
					Sebacina (from *N. nidus-avis*)	AF440657	806	90%
		b	A	KT122782	*Sebacina* (from *N. nidus-avis*)	AF440647	737	90%
		d	A	KT122783	Ascomycota (from ectomycorrhizal root)	EU624334	881	96%
	MR1	c	D	KT122784	Agaricomycetes (from forest soil)	FJ553950	292	97%
	MR2	c	D	KT122785	Pezizomycetes (from *C. damasonium*)	AY833035	905	93%
					Cadophora luteo-olivacea	DQ404349	898	96%
		e	D	KT122786	*Exophiala salmonis*	AF050274	715	92%
	MR3	c	D	KT122787	*Hymenogastraceae* (from *C. damasonium*)	AY634136	1013	94%
					Hymenogaster citrinus	EU784360	928	94%
		e	D	KT122788	*Tetracladium furcatum*	EU883432	739	90%
	MR4	b	D	KT122789	*Hymenogastraceae* (from *C. damasonium*)	AY634136	1038	97%
					Hymenogaster citrinus	EU784360	942	94%
C. longifolia	MR5	a	D	KT122767	*Tomentella* (from mycorrhizal roots)	EU668200	1170	99%
					Tomentella lilacinogrisea (from *T. cordata*)	AJ534912	989	94%
		e	D	KT122768	*Neonectria radicicola* (from mushroom)	FJ481036	937	98%
	MR6	e	D	KT122769	*Tetracladium* sp. (from *C. longifolia*)	DQ182426	985	99%
					Tetracladium maxilliforme (*P. sylvestris*)	DQ068996	985	99%
	MR7	a	C	KT122770	*Tomentella bryophila* (from beech root)	AM161534	1147	98%
					Tomentella bryophila (from sporocarp)	AJ889981	1147	98%
	MR8	Isolated	C	KT122771	Uncultured basidiomycete (from dust)	AM901992	1092	98%
					Bjerkandera adusta	AJ006672	1081	98%
	CG1	Isolated	A	KT122772	*Phlebia acerina*	AB210083	1086	98%
	CG2	b	B	KT122773	*Davidiella macrospora*	EU167591	886	98%
	CG3	a	B	KT122774	*Fusarium* sp.	EU750682	957	99%
	CG4	a	B	KT122775	*Sebacinaceae* (from mycorrhizal root)	AJ879657	1040	98%
					Sebacina incrustans	DQ520095	1037	98%

690 **Fig. 1.** Microscopical features of *C. longifolia* mycorrhizal roots: **(a)** cross-section showing
691 intracellular hyphal coils (pelotons). **(b-c)** Details of fungal pelotons in orchid root cells. **(d)** Details of
692 dark, septate and clamped hyphae emanating from pelotons. Scale bars: 500 μm (a), 200 μm (b), 30 μm
693 (c), 20 μm (d).
694
695
696
697
698
699
700
701
702
703
704
705
706
707
708
709
710
711
712
713
714
715
716
717
718
719

720 **Fig. 2.** Neighbour-joining phylogenetic tree showing the relationship between the Sebacinaceae
721 sequences obtained from the two analysed *Cephalanthera* species (*) and selected database relatives.
722 Kimura 2-parameter distances were used. Bootstrap values are based on percentages of 1000 replicates.
723 The tree was rooted with *Geastrum schmidelii* as outgroup
724
725
726
727
728
729
730
731
732
733
734
735
736
737
738
739
740
741
742
743
744
745
746
747
748
749

750 **Fig. 3.** Maximum likelihood phylogenetic tree showing the relationship between the Sebacinaceae
751 sequences obtained from the two analysed *Cephalanthera* species (*) and selected database relatives.
752 Kimura 2-parameter distances were used. Bootstrap values are based on percentages of 1000 replicates.
753 The tree was rooted with *Geastrum schmidelii* as outgroup
754